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Quadriplex real-time polymerase chain reaction (*lytA*, *mef*, *erm*, *pbp2b*^{wt}) for pneumococcal detection and assessment of antibiotic susceptibility

Velusamy Srinivasan[a], Mignon du Plessis[b], Bernard W. Beall[a], Lesley McGee[a,*]

[a]*Respiratory Diseases Branch, Division of Bacterial Disease, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA*

[b]*National Institute for Communicable Diseases (NICD), National Health Laboratory Services, Johannesburg, 2131 South Africa*

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Abstract

A quadriplex real-time polymerase chain reaction assay was developed for detecting pneumococci, penicillin susceptibility, and macrolide/lincosamide resistance. The assay was sensitive for all 4 targets (<10 copies) and correlated with antimicrobial susceptibilities in 172/180 isolates and 28/29 culture-positive clinical specimens. For 29 *lytA*-positive culture-negative specimens, the assay allowed interpretation of antimicrobial susceptibility.
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Streptococcus pneumoniae is an important human pathogen, and rapid detection and characterization from clinical specimens are critical for effective management. Culture of clinical specimens and antibiotic susceptibility testing are often slow, taking up to 48 h, and are often negative due to prior antibiotic use before sampling or autolysis of the organism. Molecular techniques applied directly to clinical material have the potential to detect the infecting organism and determine its antimicrobial susceptibility. Several real-time polymerase chain reaction (PCR) assays for the detection of pneumococci (Carvalho et al., 2007; Park et al., 2010; Suzuki et al., 2006; Tarragó et al., 2008; van Haeften et al., 2003) and pneumococcal susceptibility to penicillin (Kearns et al., 2002; Fukushima et al., 2008; Harris et al., 2008; Kumari et al., 2008) and macrolides (Kumari et al., 2008; Klomberg et al., 2005) have been described on various real-time platforms. The ability to simultaneously detect pneumococci as well as a single antimicrobial resistance determinant in clinical specimens led us to develop and validate the TaqMan quadriplex real-time PCR assay described here.

A total of 180 *S. pneumoniae* sterile-site isolates with known MICs to penicillin (PEN), erythromycin (ERY), and clindamycin (CLI) were obtained from Active Bacterial Core surveillance, a component of CDC's Emerging Infections Program. Clinical specimens included 39 cerebrospinal fluid (CSF) and 12 pleural fluid (PF) specimens previously determined to be *lytA* positive by a previously described monoplex real-time PCR assay (Carvalho et al., 2007). Twenty-nine specimens (24 CSF and 5 PF) had corresponding isolate MIC results recorded.

DNA was extracted from the bacterial isolates using Chelex 100® (Bio-Rad Laboratories, Hercules, CA, USA) and from CSF and PF specimens using the Qiagen DNA Mini kit (Qiagen, Valencia, CA, USA) with the usage of bacterial wall lytic enzymes as previously described (Carvalho et al., 2007). The *lytA* primers and probe were previously described (Carvalho et al., 2007). Primers and probes for detection of macrolide resistance (*ermB* and *mef*) and wild-type alleles of *pbp2b* typically associated with penicillin susceptibility were designed from gene sequences available in the GenBank database (Table 1). Concentrations for each primer and probe were optimized in individual assays, and optimal concentrations were used in both

* Corresponding author. Tel.: +1-404-639-0455; fax: +1-404-639-2070.
E-mail address: lmcgee@cdc.gov (L. McGee).

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Table 1
Primer and probes used in real-time PCR assays

Oligonucleotide	Sequence (5′-3′)	Nucleotide position	GenBank accession no.	References
lytA-F	ACGCAATCTAGCAGATGAAGCA	1841014	AE005672	Carvalho et al., 2007
lytA-R	TCGTGCGTTTTAATTCCAGCT	1840961		
lytA-Probe	5′-FAM-TGCCGAAAACGCTTGATACAGGGAG -3′-BHQ1	1840985		
ermB-F	CTTGGATATTCACCGAACAC	766	AB111455	This study
ermB-R	TTGGTTTAGGATGAAAGCAT	844		
ermB-Probe	5′-ROX-AAGTCTCGATTCAGCAATTGCTTAAG-3′-BHQ2	807		
mef-F	TATGGAGCTACCTGTCTGGA	291	AF227520, U83667	This study
mef-R	GGTACTAAAAGTGGCGTAACC	375		
mef-Probe	5′-HEX-CCGTAGCATTGGAACAGCTTTTC-3′- BHQ1	333		
pbp2b-F	CTGTTTGGACCATATAGGTATTT	1494906	AE007317	This study
pbp2b-R	CAATTCTTGGTATACTCAGGCT	1494976		
pbp2b-Probe	5′-Cy5-TCCAGAGCTTGGACCGCTGTGATA-3′-BHQ3	1494938		

monoplex and multiplex validation assays. Lower limits of detection were assessed with all 4 real-time PCR assays in monoplex and multiplex formats, using 10-fold serial dilutions of DNA from pneumococcal strains with known susceptibilities to PEN, ERY, and CLI. For clinical specimens that were pneumococcal culture positive, the multiplex PCR assay was performed on DNA extracted from the isolates. *mef*, *ermB*, and *pbp2b* PCR results were compared against MIC data for the corresponding cultured isolates. We determined the *pbp2b* gene sequences from isolates with MIC results that were not in agreement with the real-time assay results.

All primers and probes of the 4 monoplex assays had high sensitivity (detection limit of <10 copies/assay). In 177 of 180 test isolates that were chosen from 27 different serotypes to maximize strain diversity, the presence or absence of *pbp2b* amplification correctly corresponded to MIC values (positive signal for MICs ≤0.06 μg/mL, negative for MICs ≥0.12 μg/mL). One of the 124 PEN-nonsusceptible isolates (MIC 0.25 μg/mL) was positive for *pbp2b* amplification (Table 2). Sequence analysis results of the *pbp2b* gene from this isolate revealed the typical wild-type (*pbp2b*^{wt}) sequences corresponding to the primers and probe that, in the vast majority of recorded instances, correspond to basally penicillin-susceptible isolates with MICs <0.03 μg/mL. Further analysis revealed 10 missense substitutions near the sequence encoding the downstream KTG motif (data not shown) which conceivably conferred this low-level PEN resistance. Two of the 56 PEN-susceptible isolates were *pbp2b*-PCR negative (Table 2). One isolate had a single nucleotide change in the reverse primer region, and the second isolate had changes in both the forward (3 nucleotide substitutions) and reverse (1 substitution) primers, as well as the probe regions (1 nucleotide substitution) (data not shown).

The multiplex assay detected *ermB* and *mef* genes alone or in combination in 174 of 179 ERY nonsusceptible test isolates (Table 2) and correlated with the expected MLS_B or M-phenotype. Among these, we identified 50 (27.9%) that were positive for both *ermB* and *mef* genes. Five ERY nonsusceptible isolates were negative for either *ermB* or *mef*. Macrolide resistance in these isolates is potentially conferred through other previously described mechanisms such as mutations in ribosomal genes encoding 23S rRNA and riboproteins L4 and L22 (Tait-Kamradt et al., 2000).

Table 2
MIC ranges and genotypic profiles of *S. pneumoniae* clinical isolates validated with 4-plex real-time PCR assay

No. of isolates (n = 180)	Susceptibility patterns			Real-time PCR result				
	PEN	ERY	CLI	*lytA*	*pbp2b*	*ermB* only	*mef* only	*ermB*+ *mef*
94	NS (0.12–8)	NS (>8–>64)	NS (>0.5–>4)	94	0	46	0	48
29	NS (0.12–8)	NS (2–>32)	S (≤0.03–0.12)	29	0	0	29	0
1[a]	NS (0.25)	S (0.06)	S (0.06)	1	1	0	0	0
18[b]	S (≤0.03–0.06)	NS (1–>256)	NS (0.5–>256)	18	18	12	0	2
38	S (≤0.03–0.06)	NS (1–32)	S (≤0.03–0.12)	38	36[c]	0	37[d]	0

PEN = Penicillin; ERY = erythromycin; CLI = clindamycin; *lytA* = autolysin gene; *pbp2b* = penicillin binding protein 2b gene; *ermB* = erythromycin ribosomal methylase B gene; *mef* = macrolide efflux gene; S = susceptible; NS = nonsusceptible.

[a] Nonsusceptible to penicillin but *pbp2b* positive.

[b] Four isolates were resistant to macrolides and negative for the genes.

[c] Two isolates were susceptible to penicillin and were *pbp2b* PCR negative which had mutations in primer and probe regions.

[d] One isolate was resistant to ERY and was PCR negative for *mef* gene.